Human Retinal Pigment Epithelium Cell Changes and Expression of αB-Crystallin

A Biomarker for Retinal Pigment Epithelium Cell Change in Age-Related Macular Degeneration

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Objective: To examine changes in the retinal pigment epithelium (RPE) in eyes with age-related macular degeneration (AMD) and specifically to characterize αB-crystallin expression in RPE cells as a biomarker in this disease.

Methods: Maculae from human patients diagnosed as having AMD or from age-matched control eyes were isolated, cryosectioned, and analyzed immunohistochemically for αB-crystallin and for cell type–specific markers.

Results: In eyes with dry and wet AMD, αB-crystallin was heterogeneously expressed by a subpopulation of RPE cells in the macular region (frequently in cells adjacent to drusen) and in areas of RPE hypertrophy associated with wet AMD. In contrast, αB-crystallin was not detected at significant levels in control RPE.

Conclusion: Accompanying the formation of drusen in early-stage and late-stage AMD, RPE cells undergo change to express αB-crystallin.

Clinical Relevance: The detection of αB-crystallin in the RPE of patients with early and advanced AMD implicates this as an AMD biomarker. Sporadic expression of αB-crystallin by RPE cells localized adjacent to drusen in early AMD indicates that changes in the gene expression of RPE cells accompany early stages of the disease and introduces novel potential targets for AMD therapy.

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A GE-RELATED MACULAR DEGENERATION (AMD) is a progressive degeneration of photoreceptors and underlying retinal pigment epithelium (RPE) cells in the macula region of the retina. It is a highly prevalent disease and a major cause of blindness in the Western world.1,2 Early morphological changes include thickening of the Bruch’s membrane, the basement membrane formed by the RPE and underlying choroidal blood vessels.3 Drusen, pale excrescences of variable size, and other deposits accumulate below the RPE on Bruch’s membrane; clinical and histopathologic investigations have shown that these extracellular deposits are the hallmark of early AMD.4 As AMD advances, areas of geographic atrophy (GA) of the RPE can cause visual loss, or choroidal neovascularization can occur to cause wet, or exudative, AMD with accompanying central visual loss.

Drusen contain various lipids, polysaccharides, and glycosaminoglycans.5,5 The identity of the cells that generate these components is unclear. Direct proteomic analysis of drusen isolated by microdissection from donor eyes with and without AMD has identified a set of proteins that are more prevalent in drusen from patients with AMD.6,7 Crystallins are the most abundant proteins found in drusen from donors diagnosed as having AMD.

Crystallins, a group of small heat shock proteins, form oligomeric complexes and function as molecular chaperones protecting other proteins from denaturation and destabilization.8 They are induced by accumulation of abnormally folded proteins resulting from stress.9 Elevated expression of small heat shock proteins has been implicated in the pathogenesis of neurodegenerative disorders, including Alzheimer’s disease.10,11 Cell culture studies12,13 demonstrated that αB-crystallin might protect cells from apoptosis. In cultured RPE, induction of αB-crystallin occurs when human RPE cells are stressed by heat shock or oxidant-mediated injury, which are stresses in-
Complicated in the pathogenesis of AMD. In addition, intense light exposure increases expression of B-crystallin in photoreceptors and RPE in rats, which has been suggested to protect cells from light damage. Given these observations, we sought to study whether human RPE cells change to express B-crystallin in AMD.

METHODS

SOURCE OF TISSUE

Human ocular tissues were obtained from The Eye-Bank for Sight Restoration, Inc, New York, NY, and the National Disease Research Interchange, Philadelphia, Pa. Eyes with early, midstage, and advanced AMD and age-matched control eyes were used. Eyes with early AMD had not received a diagnosis of AMD by an ophthalmologist but had some hard macular drusen and possibly small soft drusen observed by histologic analysis and were from patients who likely did not experience AMD-related visual loss. Eyes with midstage AMD were from patients diagnosed as having AMD by an ophthalmologist and contained choroidal neovascularization or numerous hard or soft drusen, usually accompanied by scarring or GA and visual loss. Age-matched eyes that were from patients not having a diagnosis of AMD served as control specimens for this study. Twenty-three eyes were used (11 controls and 12 with AMD). Among the 11 controls, 9 eyes (5 with and 4 without nonmacular drusen) were from patients older than 60 years, while 2 eyes (without nonmacular drusen) were from patients younger than 60 years. Among the 12 eyes with AMD, 3 eyes were classified as having early AMD, 7 eyes as having midstage AMD, and 3 eyes as having advanced AMD (1 with the wet form and 2 with the dry form).

The Table summarizes the findings; 1 eye from each eye classification group is described hereinafter. Eye 1 from a 75-year-old man with early dry AMD had several hard drusen and a few soft or diffuse drusen, with minimal RPE and photore-

Table. Analysis of Eyes

<table>
<thead>
<tr>
<th>Eye Classification</th>
<th>No. of Eyes</th>
<th>Ages, y</th>
<th>Macular Drusen Detected</th>
<th>αB-Crystallin Detected in Macular RPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy control specimens</td>
<td>11</td>
<td>54, 57, 64, 67, 71, 72, 73, 74, 75, 76, 77</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Eyes with AMD Early</td>
<td>2</td>
<td>75, 79</td>
<td>++, +</td>
<td>++ + (in 1 eye)</td>
</tr>
<tr>
<td>Midstage</td>
<td>7</td>
<td>64, 75, 76, 81, 82</td>
<td>++ + (in 3 eyes), + (in 3 eyes)</td>
<td>++ + (in 2 eyes), ++ (in 2 eyes)</td>
</tr>
<tr>
<td>Advanced</td>
<td>3</td>
<td>74, 91</td>
<td>++ + (in 2 eyes), ++ (in 1 eye)</td>
<td>++ +</td>
</tr>
</tbody>
</table>

Abbreviations: AMD, age-related macular degeneration; RPE, retinal pigment epithelium; +, the level observed, with ++ + being the highest and none being the lowest.

*Eye 1 with early dry AMD described in the text.
†Eye 4 with mid-stage dry AMD described in the text.
‡Eye 3 with advanced wet AMD described in the text.
§Both eyes from this 91-year-old donor were used. Eye 2 with advanced dry AMD, described in the text, was from this individual.

Figure 1. Retinal pigment epithelium (RPE) from healthy human eyes does not express αB-crystallin, as shown in photomicrographs of healthy human retinas labeled with anti–αB-crystallin (red) and anti–αA-crystallin (green). Nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI) (blue). Higher-power views of C and G are shown in D and H, respectively, and demonstrate a lack of αB-crystallin staining in the RPE. Br indicates Bruch’s membrane; Chr, choroidal neovascularization; GCL, ganglion cell layer; INL, inner nuclear layer; and ONL, outer nuclear layer. Original magnification ×10 (A–G), ×20 (E–G), ×40 (D), and ×60 (H). The bar in G indicates 50 µm; the bar in H, 25 µm.
ceptor loss. Eye 2 from a 91-year-old man with advanced dry AMD had GA, some RPE loss, and numerous various-sized drusen. Eye 3 from a 74-year-old man with advanced wet AMD had an exudative lesion observed in macular sections. Eye 4 from an 82-year-old man with midstage dry AMD had several hard and soft drusen.

TISSUE PROCESSING AND HISTOLOGIC ANALYSIS

Eyes were procured within 12 hours of death and were fixed in a 10% formalin solution. An incision was made 3 mm posterior to the limbus, and the cornea, iris, lens, and vitreous were removed. A block of tissue approximately 6 to 8 mm² containing the macula was excised, rinsed in phosphate-buffered saline (PBS) for 2 hours, and then cryoprotected in 30% sucrose in PBS. The tissue was embedded in optimal-cutting temperature compound (Tissue-Tek; Sakura Finetek USA, Inc, Torrance, Calif) and stored at −80°C. Fourteen-micrometer-thick cryostat sections were collected for immunohistochemistry.

IMMUNOHISTOCHEMICAL ANALYSIS

Sections were rinsed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, and then treated with potassium permanganate for 30 minutes at room temperature to bleach the RPE autofluorescence, followed by treatment with oxalic acid until colorless. After washing with PBS, sections were incubated for 30 minutes in hydrogen peroxide in methanol, blocked for 30 minutes in normal serum at room temperature, and incubated with primary antibody overnight at 4°C. Primary antibodies αA-crystallin and αB-crystallin (1:2500; Biocare Medical, Concord, Calif) were used to label RPE cells. Secondary antibodies conjugated to Alexa Fluor 488/546 and Alexa Fluor 647 were used to visualize protein expression.
Here we demonstrate that αB-crystallin is expressed at elevated levels in the RPE from patients with AMD. Expression of αB-crystallin was examined using immunohistochemistry on cryostat sections obtained from 23 human eyes with different stages of AMD progression compared with aged controls (Table and Figure 1). The αB-crystallin protein was not detected in any of the RPE of age-matched controls, but was seen in the underlying choroidal layer (Table and Figure 1). The αA-crystallin was present throughout many retinal layers, as described previously.17

In contrast, αB-crystallin was found in RPE cells in all eyes with AMD examined, revealing a strong correlation between αB-crystallin expression and AMD (Table and Figures 2, 3, and 4). There were fewer αB-crystallin–positive cells in eyes with early AMD (exemplified by eye 1 with early dry AMD), and they tended to be restricted to the macular region (Figure 2A-D). In contrast, αB-crystallin–positive cells were more numerous throughout the macula and extended into the periphery in the RPE of eyes with more advanced dry AMD (exemplified by eye 2 with advanced dry AMD) (Figure 2E-F). A similar trend was observed using ABC labeling, a different visualization method for staining (data not shown).

We compared the incidence of αB-crystallin with that of αA-crystallin by double-immunostaining. The αA-crystallin was found in all the retinal layers in eyes with AMD and sometimes in the RPE layer (Figures 1, 2, and 4). Strong and distinct expression of αA-crystallin was observed in the Bruch’s membrane in the regions of GA in eye 2 with advanced dry AMD (data not shown).

The αB-crystallin was encountered frequently in RPE cells that were associated with drusen, but it was also detected in RPE cells in which no adjacent drusen were visible, as shown in Figure 3. In early AMD, αB-crystallin–positive RPE cells were found more frequently in direct contact with drusen than not (P<.001, t test), but this association is lost as AMD advances and αB-crystallin expression becomes more widespread in the RPE.

**EXPRESSION OF αB-CRYSTALLIN IN EYES WITH WET AMD**

In the eyes with wet AMD that we examined (exemplified by eye 3 with advanced wet AMD in the Table and in Figure 4), significant αB-crystallin expression was observed. Expression was strong in hypertrophic RPE cells and near choroidal neovascularization. Staining with the RPE-specific marker RPE65 showed colocalization of RPE65 and αB-crystallin in a subpopulation of cells in this layer. The αB-crystallin was also seen in the ganglion cell layer, inner nuclear layer, outer nuclear layer, and choroidal connective tissue.

**αB-CRYSTALLIN–POSITIVE RPE CELLS WERE NOT UNDERGOING CELL DEATH**

Because αB-crystallin expression has been shown to be protective against cell death and because apoptosis accompanies AMD,18 we investigated whether these αB-crystallin–positive RPE cells in eyes with AMD were undergoing apoptosis. In double-labeling experiments, RPE cells that were TUNEL (terminal deoxynucleotidyl transferase–mediated biotin-deoxyuridine 5-triphosphate nick-end labeling) positive were not αB-crystallin positive, and RPE cells that were αB-crystallin positive were not TUNEL labeled (data not shown).

**αB-CRYSTALLIN IS EXPRESSED IN A SUBPOPULATION OF CULTURED RPE CELLS**

To confirm that αB-crystallin can be expressed by RPE cells, we isolated primary RPE cells from 57- to 90-year-old cadaveric eyes, cultured these for up to 3 weeks, and stained the cultures for RPE markers and αB-crystallin expression. A subpopulation of RPE cells expressed αB-crystallin, and the incidence of positive cells was 80% at 4 days (data not shown). Hence, this biomarker is normally expressed in a subpopulation of RPE cells in culture, allowing studies of mechanisms of induction and suppression.

**COMMENT**

It is important to understand the mechanisms involved in early AMD to develop novel treatments. Here we show that
the stress-response protein αB-crystallin is a reliable biomarker of AMD-affected RPE cells from early through late stages of the disease. This indicates that individual RPE cells undergo phenotypic alteration in AMD and that the change includes expression of αB-crystallin.

We found that αB-crystallin is heterogeneously expressed in RPE cells in eyes with drusen, but we did not detect it in the RPE from healthy aged eyes. In eyes with dry AMD, αB-crystallin expression occurred more frequently in RPE cells apposing drusen and occurred less frequently in RPE cells not adjacent to visible drusen. Hence, there is a clear association between αB-crystallin expression and drusen formation in the early stages of AMD. The function of αB-crystallin in AMD is unknown; however, it may help protect stressed RPE cells from cell death.

In conclusion, the detection of αB-crystallin in the RPE throughout AMD progression opens up new avenues for exploring the changes in RPE cells that accompany AMD. The RPE cells change expression in an individual cell-by-cell manner rather than as a generalized process diffusely affecting the entire RPE layer. This leads us to hypothesize that the mechanism of AMD includes the activation of cell-intrinsic programs to alter individual RPE cells. Further studies of such changes in RPE cell phenotype will aid in our understanding of the mechanisms of drusen formation and, ultimately, the pathogenesis of AMD.

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**Archives Web Quiz Winner**

Congratulations to the winner of our December quiz, Christos Christakopoulos, MD, Department of Ophthalmology, Eye Clinic of Aalborg Hospital, Aarhus University, Denmark. The correct answer to our December challenge was metastatic malignancy. For a complete discussion of this case, see the Clinicopathologic Reports, Case Reports, and Small Case Series section in the December ARCHIVES (Andreoli CM, Husain D, Davis TS, Loewenstein JL. Chorioretinal changes heralding metastatic malignancy. *Arch Ophthalmol.* 2006;124:1790-1792).

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